"*In vitro* Rapid Mass Multiplication and Molecular Validation of *Rheum emodi*''

SYNOPSIS SUBMITTED

FOR

Ph.D. DEGREE

IN

BIOTECHNOLOGY

BY

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BACKGROUND

The Northern Himalayas are flourishing with a wide range of highly demanded medicinal plants. About 30,000 medicinal plant species are there in world in which 8000 species are found in India [1]. For majority of world's population, medicinal plants play a vital source as life saving drug, especially in India because India has a plant based ethno medical tradition. Around 32600 tones of medicinal plants in a year is exported by India[2], This increased the demand of herbal medicine which brings the medicinal plants to a stage of near threatened to critically endangered. This suggest that the there is an urgent need to develop or design strategies or put some quick steps for the conservation of medicinally important plants.

In nature, the conventional propagation of medicinal plants through vegetative propagation or seed propagation is slow and this process is very much hampered by the environmental conditions as well as poor germination power of seed even the death of seedlings at 2- leaved stage. During last two decades, plant tissue culture through *in-vitro* regeneration has been increasingly applied for rapid mass multiplication or clonal propagation of highly valuable, endangered species of medicinally important plants with different degree of success.

Rheum emodi, which is commonly known as Indian or Himalayan rhubarb, is an endangered species of family Polygonaceae. It is a sturdy perennial herb distributed through Himalayas extending from Kashmir to Kumaon. In Sanskrit, Rheum emodi is known by name Revatchini, Revandchini in Unani and in Ayurvedic system this species is mentioned as Amlaparni, Pitamuuli[3]. Rhubarb constitutes around 60 species around the world and among them 10 species are belong to Indian Himalayan regions [4]. *Rheum emodi* grows in temperate and sub tropical regions of northern Himalayas which cover the countries like India, Nepal, Bhutan and China. Endemic to northern Himalayas, *Rheum emodi* is distributed in the altitudes of ranging from 2800 to 3800 m. In Himalayas of Garhwal, this species is generally found in Alpine zone having rocky soil in 2800 to 3600 m altitudes [5].

Its morphological characteristics is described as, it has a height ranging from 1.5-3.0 m, with broad leaves and thick stem. This perennial herb has small flowers of purple to pale red colour with 3 mm diameter. Roots are dark brown, strong and thick. Rhizomes are dull orange to yellowish brown colored with a length of 6-12 inches [6].

From the ancient period of time, rhubarb is used widely in many medicine system viz., Unani, Ayurvedic, Chinese etc. Indian Rhubarb, used as purgative, stomachic, astringent tonic, and also effective in many skin diseases [7]. *Rheum emodi* can also be taken in piles, lumbago, jaundice, and diarrhea. *Rheum emodi* and its preparations are very effective in urogenital system and respiratory system related problems [8]. Major phytoconstitutes of Rhubarb are hydroxyantraquinone derivatives like physicon, chrysophanol emodin, aloe- emodin, rhein and their glycosides[9], which are effective as in many biological activities including anti oxidant [10] nephroprotective activities[11], antifungal, anti microbial, anti-inflammatory properties[12]. Along with the medicinal use, plant's roots are used as natural dye in cosmetics, textiles and as food colouring agent [13]. Besides this, its leaves are used for preparing vegetables and salads [14]. In view of continued use of *Rheum emodi* in modern as well as traditional system of medicine, this species come in the stage of endangered category. Therefore tissue culture techniques like micropropagation, *in-vitro* regeneration is needed to introduce for the conservation of this medicinal herb even in their natural habitat. This enables the mass propagation of this valuable plant, by which further requirement of extraction of active compound can be fulfilled.

In recent years, molecular approaches are being considered as alternatives for conservation and improvement of various plants through genetic profiling. Different markers have been developed for the authentication of plant species. PCR based markers like RAPD/SSR involves *in-vitro* amplification of particular sequences by using thermostable DNA polymerase enzyme and specific oligonucleotide primers. DNA marker techniques like RAPD, AFLP, ISSR, SSR are used for genetic studies such as linkage map construction, evaluation of genetic diversity and population structure, even for DNA fingerprinting these DNA markers are used [15]. Therefore, present study has an aim to assess the *in-vitro* propagated *Rheum emodi* through molecular marker for the pure germplasm and somaclonal variation.

In today's world consumption of herbal medicines are increasing worldwide. Their increase use and harvesting in indefinite amount are the main cause for their loss of genetic diversity and habitat destruction. So, for fulfilling the increase demand of the medicinally important plants, different biotechnological tools are used such as genetic transformation or tissue culture technique, which help in the multiplication, enhancing the genetics of medicinal plants, even the product as high quality medicines which are derived from plants[16].

The *Rheum* species are rapidly declining in population in their habitat. A report of IUCN (International Union of Natural and Natural Resources) indicates that among the 10 species of vascular plants, 1 is in the endangered list and rheum emodi makes its place in top of that list [17].This leads to the decrease in gene flow and gene

erosion in upcoming years. There are many possible causes for the species going to the endangered category. Some of them may be lack of conservation of species, lack of cultivation, environment effect or climate change etc. In a study, some facts proved that in last 15 years, threat status of *Rheum emodi* increased, lack of awareness among people for cultivation increases the burden on natural reserves for conservation of the species [18].

As the *Rheum* species is highly demanded because of medicinal use and endangered status so the requirement for conservation is increased. Seed age in *Rheum emodi* is very short, even one year old seeds show very poor germination. The treatment with GA_3 of 50ppm shows only 25% of germination, and the higher concentration of GA3 as 100ppm was proved to be inhibitory in *Rheum emodi* seed's germination. This shows that the age of seeds has direct effect on their germination [19].

As seed viability and rate of germination are low, thus seed propagation alone cannot provide quality planting material for sustainable use. Mass propagation is only possible through *in vitro* regeneration, which is a powerful tool for tissue culture. Thus the regeneration becomes very much important.

In-vitro propagation also referred as true-to-type propagation, this use *in-vitro* culture techniques for selected genotypes. *In-vitro* propagation technique play an important role for producing virus free plants, high quality plant based medicines etc. Different factors are responsible for the well developed protocol of *in-vitro* regeneration such as the age and source of explants, establishment of aseptic culture, efficient acclimatization of tissue culture raised plants. There are several medicinal plants whose *in-vitro* propagation has been achieved by using different tissue culturing techniques [20].

Propagation of Indian rhubarb can be done by using shoot-tip and leaf as explants. By using MS media with different combination of phytohormones like BAP (6-benzylaminopurine) as 2mg/l that gave multiple shooting by using excised leaves as explants and IBA (Indole-3- butyric acid) as 1mg/l induce roots in 4 weeks. Some explants failed to induce multiple shoots when submerged in liquid culture and turned brown. These regenerated plants also show same chromosome number as the parent plants [21]. This study provides the outline for the requirement of hormonal composition to obtain higher number of plantlets in lesser period of time with good rate of growth.

Previous worker Malik, in a study on regeneration of *Rheum emodi* Wall towards conservation of an endangered medicinal plant species, shows that BAP as 10µm and IBA as 5µm on MS media also give the multiple shooting. Leaves shows Organogenesis when the cultures remained for 60 days on same media without sub-

culturing, no. of proliferating adventitious buds are 6-7 buds per leaf; stress could be the reason for the same[13].

Majorly found chemical constituent of *Rheum emodi* are Anthraquinones, Stibenes, Oxanthrone's derivatives, flavanoid, carbohydrate and oxalic acid. Anthraquinone which is a polycyclic aromatic hydrocarbon, naturally found in different plant family like Polygonaceae, Fabaceae, Liliaceae and Rubiaceae. Anthraquinone found in *Rheum emodi* are emodin, aloe-emodin, rhein, physicon, chrysophanol. These anthraquinones inhibit the proliferation of many human cancer cell lines **[1]**. Emodin induces apoptotic cell death in human breast cancer cell [22] and inhibits proliferation of human gastric cancer cell line. Aloe-emodin works as anti proliferating agent of carcinoma cell line CH27 of human lung squamous cell [23].

Another worker Hussain, investigate the antibacterial, antifungal as well as insecticidal activities of *Rheum australe*. Study conclude that the *Rheum austral* has a highest antibacterial activity against *E. coli* and *E. aerogenes* while the crude extract of rhizome has a antifungal activity against *F. solani* and *A. niger* and as a insecticidal it showed high mortality rate against *C. analis*[24].

Plantlets raised by *in-vitro* culture have low amount of ABA but its content rose up during the stress condition and this also helps in acclimatization of plants. When propagated plantlets are transferred from *in-vitro* to ex-vivo environment they face stress with high mortality rate. So for cope up this the biotization is done in which resistance is induced through inoculants [25]. Successful hardenings were seen in case of tea plants raised through the tissue culture when treated with suspension cells of genus *Bacillus* and *Pseudomonas* [26]. The bacteria associated with rhizosphere help in enhancing the growth and survival rate of in vitro raised plants. These bacteria are also known as PGPR (plant growth promoting regulators) [27].

The basic chromosome no. in *Rheum* is x=11 and its species have different ploidy level ranging from diploid (2n=2x=22) to hexaploid (2n= 6x= 66). Gilmore et al. (2014) generated sequences of 97 dinucleotide by short read DNA sequencing and filtering for rhubarb. Their data is available at NCBI, 2012. For rhubarb fingerprinting, they found that 25 SSRs were very effective. These SSRs can be used also for population structure of wild rhubarb like *R. officinale, R. tanguticum and R. palmatus*. These short read sequencing can also be used for the development of SSR markers [15].

Variability at the genetic level that includes the alleles is collectively known as genetic diversity. It also refers to the diversity present in the germplasm of any species. Different worldwide studies on genetic diversity in *Rheum* species with small sample size using SSR marker [28] and ISSR markers [29] were done in past few years. In India, genetic diversity is measured in *Rheum* species of different geographical

population using ISSR markers in Kashmir Himalaya. In this report, 3 different species, Rheum *emodi, Rheum speciforme, Rheum webbianum* were studied in which *Rheum emodi* has showed lowest polymorphism of 78.8 %, while *Rheum speciforme* has 83.6% and *Rheum webbianum* has showed the highest percentage i.e. 86.8% of polymorphic fragment [30]. These types of studies have shown the path for future work on genetic diversity and population structure of *Rheum* species. The ISSR primer which was used in above mentioned study can be useful for the authentication, validation as well as for characterization of *Rheum* germplasm in future study. They also provide the important data for conservation and management of the Rheum species in Himalaya regions.

OBJECTIVES OF THE STUDY

For the proposed study, objectives are as follows:

1. *In-vitro* regeneration of *Rheum emodi.*

- To standardize the sterilization techniques.
- To optimize the media components and plant hormones using Box- Behnken method for optimum growth of plants.
- To develop suitable formulation of media for *in-vitro* shooting and rooting.
- Establishing suitable conditions for acclimatization and hardening of plants raised from tissue culture technique.

2. Screening of somaclonal variation using molecular markers.

3. Production of synthetic seeds and estimation of their viability and germination potential.

Work Plan and Methodology of the Proposed Study:

1st Year

1. Survey for site selection:
Surveys of various geographical locations will be organized and places were screened for collecting explants in Uttarakhand.

2. Explants collection:
From healthy and disease-free plants, explants will be collected.

3. Standardization of sterilization techniques:
Different sterilization techniques were standardization of explants like ethanol, mercuric chloride and sodium hypochlorite treatment.

4. Establishment of culture and its statistical analysis:
Appropriate cultures will be established by shoot apices induction/ callus induction for regeneration of plants. Statistical optimization of nutrients of tissue culture media will be done by using RSM.

2nd year

1. Clonal propagation of cultured *Rheum* species:
Rate of clonal propagation will be checked in culturing media having different composition of plant hormones.

2. Encapsulation and germination of somatic embryos:
Somatic embryos will be encapsulated in sodium alginate and calcium nitrate for production of synthetic seeds and will be kept for germination at some definite interval to estimate their viability.

3. Standardization of *In-vitro* rooting of cultured plants:
To find out best formulation, different auxin composition in media will be standardized for successful *in-vitro* rooting.

4. Genomic DNA isolation:
After clonal multiplication, genomic DNA will be isolate using CTAB method with some modification.

5. Standardization of PCR technique:
For amplification of genes, different composition of PCR like polymerase enzyme, extension time, Mg2+ concentration.

3rd year

1. SSR/RAPD analysis of tissue cultured raised plants:
With distinct selected primers, screening of different individuals by using SSR/RAPD analysis will be perform.

2. Hardening and acclimatization of *In vitro* plants:
For hardening, exposure of microbiome will be given to the tissue cultured raised plants.

3. Statistical analysis:
In all experiments, triplets will be used and the collected data are subjected to Analysis of Variance (ANOVA), for finding the significance of differences observed in tissue cultured plant of *Rheum emodi*.

EXPECTED OUTCOMES

1. Seed viability and rate of germination are low and quality seed production is a laborious task. Thus developed and validated protocol will help in the mass production of *Rheum emodi*.

2. Batch with variability will be excluded from our long term plant propagation and improvement programs thus helping saving unnecessary time, labour and also money.

3. Through RAPD/ISSR profiling of *Rheum emodi*, initiation of conservation steps in natural habitat and mass production of this species is increased.

4. Synthetic seeds can be used as a substitute to the natural seeds. As production of synthetic seeds is not time or season dependent, thus can be available throughout the year. It can also reduce the dormancy period.

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